# A proteogenomic view on antibiotic resistance in pathogenic Enterobacter species

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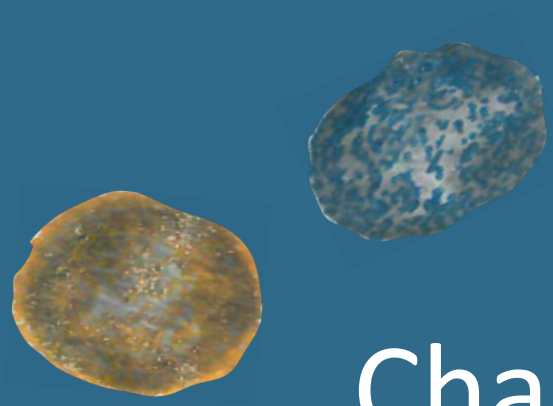
*DOI:*
10.33612/diss.103413360



# Chapter 4

## Multi-omics analyses of clinical isolates belonging to the *Enterobacter cloacae* complex uncover differences in expression of determinants for antibiotic resistance and virulence

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Manuscript to be submitted

## Abstract

Bacterial species belonging to the *Enterobacter cloacae* complex occupy many different ecological niches, including the human body where they can cause severe infections. The present study was aimed at distinguishing particular features of '*E. cloacae*' isolates from patients at the University Medical Center Groningen that were remarkable in terms of resistance to carbapenems or association with bone infections. To distinguish these isolates, a combined genomics and proteomics approach was applied. First, comparison of the respective genome sequences showed that the isolates with different clinical presentation belonged to different *Enterobacter* species. The carbapenem resistant gut isolate was identified as *E. roggenkampii*, whereas another gut isolate was identified as *E. hormaechei* subsp. *hoffmannii*. The bone isolates belong to different *E. hormaechei* subspecies or *E. cloacae*. This implies a different evolutionary history of the respective isolates, which might explain their different niche preference in the human body. The subsequent proteome analysis showed that the isolates associated with bone infections produced proteins related to type VI secretion systems, which are generally implicated in virulence, and proteins related to flagellar function for bacterial motility. Consistent with these proteome data, the bone isolates contained two to three type III secretion systems for flagellar biogenesis, and two to three type VI secretion systems. The genes for these systems were all integrated between tRNA-encoding genes, suggestive of acquisition by horizontal gene transfer. On the other hand, the carbapenem-resistant *E. roggenkampii* prominently expressed proteins related to antibiotic resistance and resistance to copper. Together, these observations support the view that the investigated isolates, all belonging to the *E. cloacae* complex, have evolved in different directions to optimally adapt to different niches with different selective pressures.

## Introduction

The genus *Enterobacter* includes rod-shaped, non-sporulating, motile, facultative anaerobic, Gram-negative bacteria belonging to the family of *Enterobacteriaceae*. Representative species of this genus, like *Enterobacter cloacae*, can be isolated from a wide variety of environments, ranging from the soil and sewage to the human gastrointestinal tract. In fact, *E. cloacae* is frequently identified among the human gut microbiota. In recent years, *E. cloacae* is increasingly encountered as a causative agent of opportunistic infections, especially in intensive care units [1]. For instance, *E. cloacae* has been implicated in skin and soft tissue infections, respiratory and urinary tract infections [2,3,] intra-abdominal infections, bacteremia, endocarditis, septic arthritis, and osteomyelitis in immunocompromised patients [4,5]. Most *E. cloacae* isolates implicated in infections are still sensitive to antibiotics, but also among recent *E. cloacae* isolates a trend towards increased antibiotic resistance is emerging [6].

The 'species' *E. cloacae* was originally referred to as *Bacillus cloacae* in 1890 but, after various name changes, the name *E. cloacae* was finally coined in 1960 [7]. Nonetheless, when studying '*E. cloacae*', it should be noted that this species is quite diverse as underscored by Multi Locus Sequence Analysis and Hsp60 typing [8]. In contrast, different *E. cloacae* isolates cannot be reliably distinguished by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-TOF MS) or 16s rRNA typing [9,10]. This makes a precise distinction of *E. cloacae* isolates difficult for the routine diagnostic procedure. In fact, this difficulty has led to multiple taxonomy changes that took place since the definition of *E. cloacae* as a species in 1960 [7] and, today, it is probably more appropriate to refer to the '*E. cloacae* complex'.

Recently, we identified a carbapenem resistant isolate belonging to the *E. cloacae* complex, which had the sequence type (ST) 232 and which expressed a cephalosporinase with carbapenemase activity [11]. Based on its whole-genome sequence, we subsequently specified this isolate more precisely as *E. roggenkampii*. Although carbapenem resistance is still rare in *E. cloacae* and its close relatives, the identification of this resistance phenotype in *E. roggenkampii* is highly worrisome in view of the fact that bacteria belonging to the *E. cloacae* complex can cause serious invasive disease. Therefore, we wanted to pinpoint to

what extent the *E. roggenkampii* ST232 isolate differs from other clinical isolates belonging to the *E. cloacae* complex. To this end, we followed a multi-omics approach, which highlights particular differences between the investigated *E. roggenkampii* ST232 isolate, the *E. cloacae* type strain ATCC 13047, and isolates of the *E. cloacae* complex that were associated with human bone infections. Notably, the latter clinical association was unexpected at the time these isolates were identified but, today, isolates belonging to the *E. cloacae* complex are in fact increasingly implicated in bone infections [5].

## Results and Discussion

### Phylogenetic differences in *Enterobacter cloacae* isolates

Altogether, seven '*E. cloacae*' isolates collected from patients at the University Medical Center Groningen, the Netherlands, were included in the present study, along with the ATCC 13047 *E. cloacae* type strain. Five of these strains were isolated from bone infections, while two other isolates were obtained from neonatal rectal swabs. One of the latter two isolates was previously characterized as *E. roggenkampii*, and found to be resistant to carbapenems. The other clinical isolates showed insignificant antibiotic resistance levels (Supplementary Table S1). Since the distinction of species belonging to the *E. cloacae* complex is difficult, we decided to characterize the selected clinical isolates by whole-genome sequencing, using a hybrid assembly approach that combined short- and long-read sequencing data. Next, the taxonomical distance between the selected isolates was assessed by phylogenetic analyses, which included 36 publicly available genome sequences of different species belonging to the *E. cloacae* complex (Supplementary Data S1). The neighbor joining tree based on the whole-genome sequences is presented in Figure 1. Importantly, this analysis showed that the selected '*E. cloacae´* study isolates belong to different species and sub-species. In particular, the isolates 61202 and 55602, respectively associated with upper arm and heel bone infections, are *Enterobacter hormaechei* subsp. *steigerwaltii* and represent a relatively recently discovered subspecies of *Enterobacter hormaechei*. The isolate 44101, which was associated with a heel bone infection, clustered together with the *E. cloacae* type strain

ATCC 13047, and it was therefore identified as *E. cloacae* subsp. *cloacae*. The isolate 99101, associated with a tibia infection, clustered with members of the *E. cloacae* complex clade S_ND22. The isolate 88701, which was derived from fluid collected from a hip region, clustered together with members of the *E. cloacae* complex clade S_GN06232. Lastly, the gut isolate 141024K was identified as *E. hormaechei* subsp. *hoffmannii*. As previously shown, the already characterized carbapenem resistant *E. roggenkampii* isolate (339389L) was grouped together with other known *E. roggenkampii* strains in the present phylogenetic analysis.

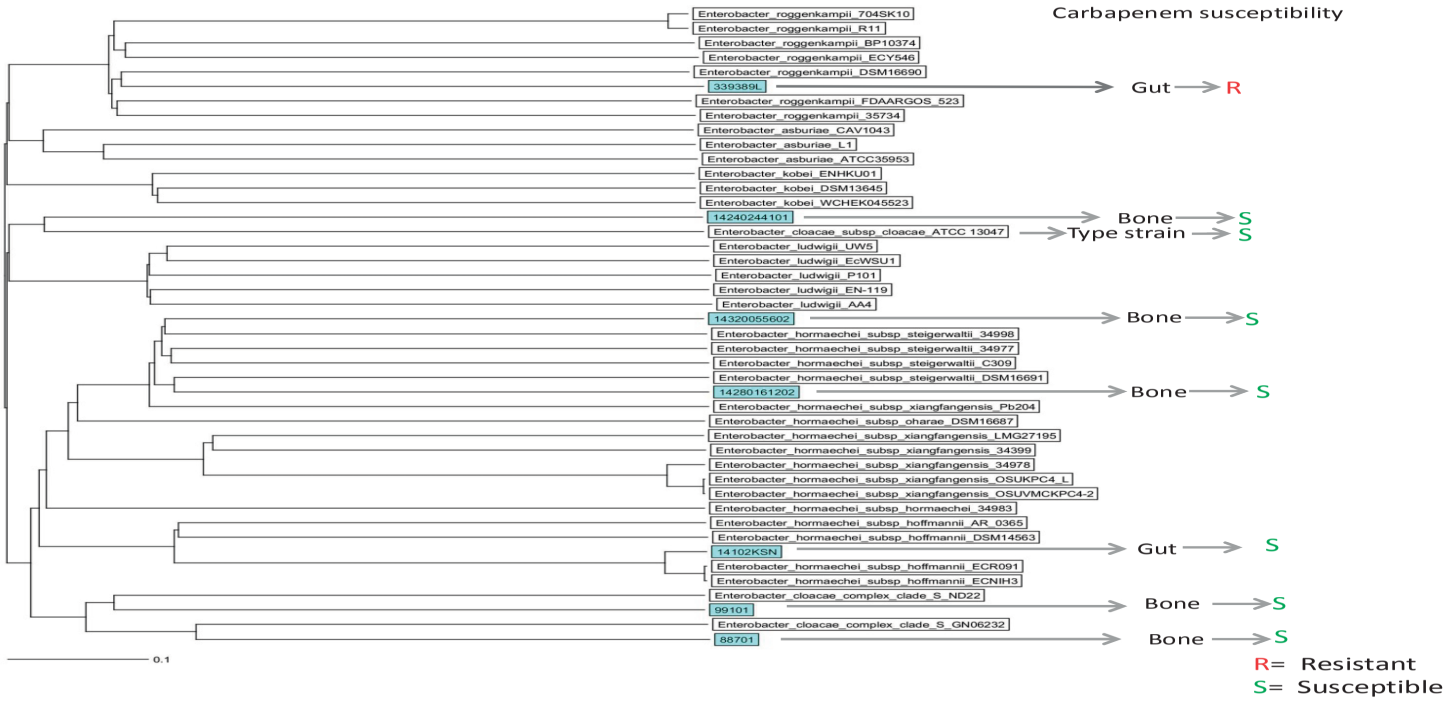


Figure 1. Whole-genome based phylogeny based on comparative genome Multi-Locus Sequence Typing (cgMLST) of seven study isolates belonging to the *E. cloacae* complex, and the *E. cloacae* type strain ATCC 13047. A neighbor joining tree was constructed based on the genome sequences of the study isolates, the *E. cloacae* type strain and representative publicly available genome sequences of bacteria belonging to the *E. cloacae* complex using Ridom SeqSphere+ version 5.1.0. The tree highlights the different clades belonging to the *E. cloacae* complex. The resistance (R) or susceptibility (S) to carbapenems of the study isolates is indicated. The scale bar indicates the genetic divergence relevant to branch length and is based on units of the number of nucleotide differences per site across 794 loci.

**Comparative proteome analysis differentiates the carbapenem resistant *E. roggenkampii* gut isolate from isolates implicated in bone infections**

Both the carbapenem resistance and the association with bone infections were unexpected observations for isolates from the *E. cloacae* complex. We therefore asked the question whether the respective isolates might present differences in their protein expression profiles that cannot be inferred from the genome sequence. To this end, the seven clinical isolates as well as the *E. cloacae* type strain ATCC 13047 were grown in BHI broth, harvested at the same growth stage, and subjected to proteome analysis. This led to the identification of 979 proteins in total (Supplementary Table S2). Roughly 25% of the identified proteins were detected in all investigated isolates, thus representing their core proteome (Figure 2). By analyzing Clusters of Orthologous Genes (COG) categories and functions based on Gene Ontology (GO), it was found that the largest categories of identified proteins of the core proteome are related to translation, ribosomal structure and biogenesis, energy production and conversion, amino acid transport and metabolism, carbohydrate transport and metabolism, and post-translational modification, protein turnover and chaperones (Supplementary Table S3). Importantly, for individual isolates additional proteins were identified that were shared with a limited number of other isolates, or that were unique for the respective isolate (Figure 2).

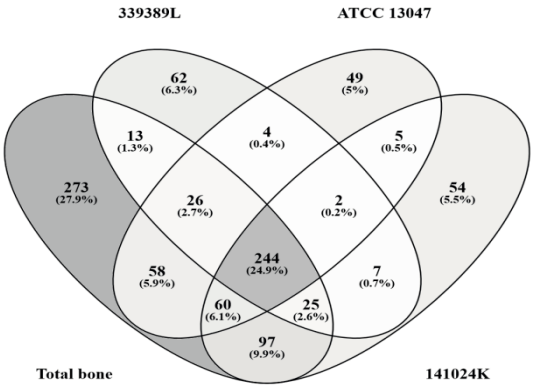


Figure 2. Venn diagram showing the numbers of uniquely and commonly identified proteins of the seven study isolates belonging to the *E. cloacae* complex, and of the *E. cloacae* ATCC 13047 type strain. For this comparison, the 796 proteins identified in isolates associated with bone infections were included as one group marked as 'total bone'.

To approximate potentially specific protein features of groups of investigated isolates, i.e. the five bone infection-related isolates *versus* the carbapenem resistant *E. roggenkampii* isolate (339389L), the gut isolate 141024K, or the ATCC 13047 type strain, the identified proteins of each group and their annotated functions were compared (Supplemental Table S3). This unveiled the proteins that were unique for the respective groups, at least under the present standardized experimental growth conditions, which are different from the conditions at the respective sites of isolation. It should be noted that many of these unique proteins are related to bacterial metabolism. However, certain features were characteristic for particular isolates. Thus, for the carbapenem resistant *E. roggenkampii* isolate, the AmpC-type β-lactamase responsible for the observed carbapenem resistance stood out among the list of identified proteins, and the same was true for several proteins involved in copper homeostasis. These proteins were neither detectable in the *E. cloacae* type strain, the carbapenem susceptible gut isolate, nor the isolates related to bone infection (Supplemental Table S3). On the other

hand, the latter group of isolates uniquely featured various flagellar proteins involved in bacterial motility (FliC, FliD, FliG, FlgK), a fimbrial protein involved in adherence to surfaces and tissues (SfmA), proteins involved in type VI secretion systems (EvpB, ImpA, ImpC, ImpE) and known effector proteins that have been implicated in bacterial virulence (ImpD, MviM). In particular, given the clinical presentation of the isolates associated with bone infections, the identification of type VI secretion-related proteins and effectors, which are known to be associated with virulence is very intriguing. Yet, while typical for the investigated isolates, it has to be noted here that these virulence-related proteins are not represented in all individual isolates associated with bone infections, but rather are expressed by at least one of the five respective isolates (Supplemental Table S3)[8,6]. To reconcile these observations with the genome sequences, we inspected the occurrence of flagellar and type VI secretion related genes in the genomes of the investigated isolates. This showed that they contained two to three type III secretion systems for flagellar biogenesis, and two to three type VI secretion systems (Table 1). As exemplified for isolate 99101, the respective genes are located at different chromosomal positions (Figure 3). Furthermore, the genes for the identified type III and type VI systems are all integrated between tRNA-encoding genes as specified in Table 1, which is suggestive of acquisition by horizontal gene transfer. It should be noted, however, that the tRNA genes are not directly flanking the secretion systems.

Table 1. Identified type III and type VI secretion systems in the investigated isolates belonging to the *E. cloacae* complex.

| | bone infection isolates | | | | | Type strain and gut isolates | | |
|---|---|---|---|---|---|---|---|---|
| **Identified loci for type III or type VI secretion systems and flanking tRNA genes** | **99101** | **88701** | **55602** | **61202** | **44101** | **ATCC 13047** | **141024K** | **339389L** |
| (Leu-CAA)T3SS(Leu-CAG) | + | + | - | - | - | - | - | - |
| (Ser-GGA)T3SS(Ser-GGA) | + | + | - | - | - | - | - | - |
| (Gly-GCC)T3SS(Asn-GGT) | + | + | + | - | + | - | - | + |
| (Thr-TGT)T3SS(Gly-GCC) | - | - | - | - | - | + | - | - |
| (Ser-GGA)T3SS(Val-GAC) | - | - | + | + | + | + | + | - |
| (Asn-GGT)T3SS(Asn-GGT) | - | - | - | - | - | + | + | - |
| (Tyr-GTA)T3SS(Leu-CAA) | - | - | + | - | - | - | - | - |
| (Glu-TCC)T3SS(Arg-ACG) | - | - | - | + | - | - | - | - |
| (Phe-GAA)T3SS(Asn-GGT) | - | - | - | + | - | - | - | - |
| (Arg-TCT)T6SS(Tyr-GTA) | - | + | - | - | - | - | - | - |
| (Tyr-GTA)T6SS(Leu-TAA) | + | + | + | + | + | + | - | + |
| (Phe-GAA)T6SS(Met-CAT) | + | + | - | - | - | - | - | - |
| (Val-GAC)T6SS(Tyr-GTA) | - | - | + | + | - | + | + | - |
| (Ser-GGA)T6SS(Val-GAC) | - | - | - | - | + | - | + | - |
| (Glu-TTC)T6SS(Thr-GGT) | - | - | - | - | - | - | - | + |

+, identified; -, absent; T3SS, type III secretion system; T6SS, type VI secretion s

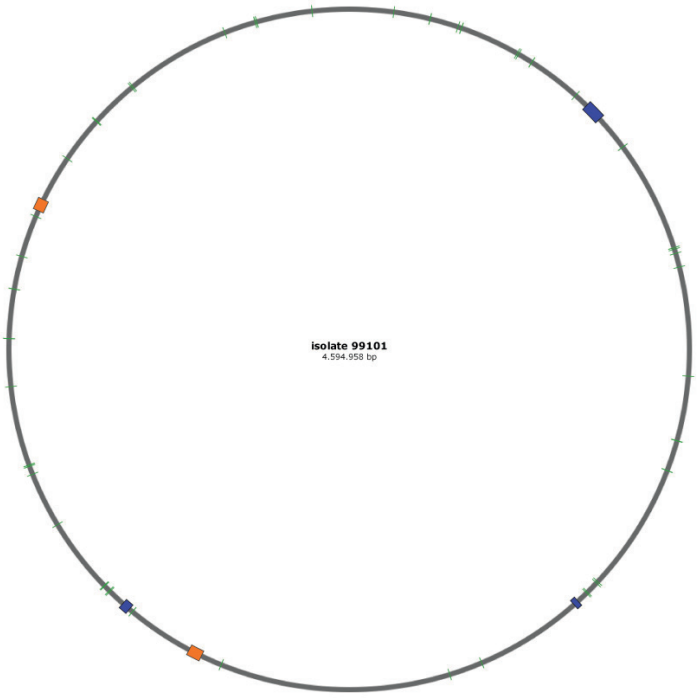




Figure 3. Schematic representation of the chromosomal localization of genes encoding type III and type Vi secretion systems in study isolate 99101. The secretion systems were detected with the online tool T346Hunter [12]. The orange boxes represent type VI secretion systems and the blue boxes represent flagellar type III secretion systems. In green, the tRNA genes are shown.

## Conclusion

Altogether, the here presented study highlights the diversity of bacteria belonging to the *E. cloacae* complex, both at the genomic and proteomic levels. This diversity is seemingly reflected in their clinical presentation. It should be noted that, like the investigated carbapenem resistant *E. roggenkampii* isolate, all other investigated isolates carry *ampC* type cephalosporinase genes. However, contrary to the carbapenem resistant isolate, the other investigated isolates do not seem to express these resistance genes under the standardized experimental

conditions of the present study. Conversely, the carbapenem resistant *E. roggenkampii* isolate carries genes for two type VI secretion systems, but it did seemingly not express these genes under the tested conditions, whereas isolates associated with bone infections did express proteins of type VI secretion systems. These observations add fuel to the assumption that the different isolates have been subject to different selective pressures that either led to resistance against carbapenems, or the expression of a secretion system that has been associated with bacterial virulence. At present we do not know to what extent these different phenotypical presentations can be related to different clades within the *E. cloacae* cluster. However, this is definitely something worth investigating in a future study because, if the different clades can indeed be associated with either high drug resistance or virulence, the position of '*E. cloacae*' isolates in the phylogeny of this complex might be applicable for approximating the risk for resistance against carbapenems, pathogenicity, or both. In this respect, it would be really interesting to screen a collection of different *E. roggenkampii* isolates for possible carbapenem resistance.

## Materials and Methods

### Bacterial isolates

The bacterial isolates used in this study and their antibiotic resistance are listed in Supplementary Table 1. The initial species determination was performed by MALDI-TOF MS, using a Bruker Microflex (Bruker Corportation, Billerica, USA), showing that they all belonged to the *E. cloacae* complex.

### DNA sequence analyses

Nanopore sequencing. Bacteria were grown overnight at 37°C on Blood Agar (BA) plates. Then single colonies were picked for overnight culture at 37°C on BA plates again. For DNA extraction the DNeasy UltraClean Microbial Kit (Qiagen) was used with minor modifications. A 10 µl-loopful of bacteria was directly transferred into a tube with microbeads and microbeads solution. The incubation period was prolonged to 20 min, instead of 5 min. The quality and quantity of isolated DNA was determined using a Qubit® 2.0 fluorometer (ThermoFisher

Scientific), an Agilent Tapestation 2200 (Agilent) and a NanoDrop (ThermoFisher Scientific). Libraries were prepared without shearing to maximise sequencing read length. The library was prepared using the 2D ligation sequencing kit (SQK-LSK208). The protocol for 2D ligation sequencing kit was followed as described by the manufacturer. The final library was loaded onto a FLO-MIN106 R9.4 flow cell. The run was performed on a MinION device using the NC_48Hr_Sequencing_Run_FLO-MIN107_SQKLSK208 protocol with 976 available pores (464, 314, 161 and 37 pores per group). The run proceeded for the full 48 hours. Base calling was performed after the run, using Albacore v1.2.2 (Nanopore) with the r94_250bps_2d.cfg workflow. Lastly, the quality of the data was analysed with Poretools v0.6.0 [13] and the fast5 files were transformed into a fastq file.

Illumina sequencing. Total DNA extraction for whole-genome sequencing was performed directly from colonies of the respective isolates using the Ultraclean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, US) according to the manufacturer's protocol. DNA concentrations were determined using a Qubit® 2.0 fluorometer and the dsDNA HS and/or BR assay kit (Life technologies, Carlsbad, CA, US). Subsequently, DNA libraries were prepared using the Nextera XT v3 kit (Illumina, San Diego, CA, US) according to the manufacturer's instructions. Sequence analysis was performed with an Illumina Miseq System generating paired-end reads of 300 bp as described previously [14]. *De novo* assembly of paired-end reads was performed using CLC Genomics Workbench v8.5.3 (QIAGEN, Hilden, Germany) after quality trimming (Qs ≥ 20) establishing a word size of 30. The raw WGS datasets generated in the current study are available in the European Nucleotide Archive (ENA) repository under Bioproject PRJEB22119 (http://www.ebi.ac.uk/ena/) or are available on request.

Hybrid assembly. The hybrid assembly of the Illumina short reads and the MinION long reads of the different isolates was performed using SPAdes version 3.10.1 (http://bioinf.spbau.ru/en), or Unicycler version 0.4.1 [15]. Bandage v0.8.1 was used to visualize the Unicycler assembly graphs [16]. The resulting assemblies are submitted to NCBI under accession number CP026536 or are available on request. Sequence analyses were performed using the rapid annotation using subsystem technology (RAST) server 4.0 [17].

**Phylogenetic analyses**

A neighbor joining tree was constructed based on the genome sequences of the study isolates, the *E. cloacae* type strain ATCC 13047 and 36 representative publicly available genome sequences of bacteria belonging to the *E. cloacae* complex using Ridom SeqSphere+ version 5.1.0. (Münster, Germany) with default settings [18] The relatedness of the different genomes was analyzed using a local *ad hoc* cgMLST scheme based on 794 targets (749259 bases) and 3647 targets were used as accessory targets (3493464 bases), and the missing values were ignored (see Supplemental Data S1).

**Proteome analyses**

All *Enterobacter* isolates were cultured in triplicate in brain heart infusion broth (BHI; Oxoid, Basingstoke, UK) at 37 °C with vigorous shaking at 250 rpm. For sample preparation, cells were collected in the mid-exponential and stationary growth phases by centrifugation and disrupted by bead-beating with glass beads (~0.1 mm diameter) in a Precellys 24 homogenisator (Bertin Technologies, France) as described previously [19,20]. Glass beads and cell debris were removed by centrifugation (21,000 × g, 10 min, 4 °C). The cell extract protein fraction was prepared and analyzed by LC-MS/MS using an Orbitrap Velos Pro mass spectrometer (ThermoFisher, Waltham, MA USA) as described previously [21] . Briefly, proteins were concentrated with Strataclean beads, subsequently reduced and alkylated, digested with trypsin and then purified through StageTip purification. Desalted peptides were loaded on an EASY-nLC™ II nano-flow LC system (ThermoFisher) with 10 µl buffer A (0.1% (v/v) acetic acid) and a constant flow rate of 0.5 µL/min. Afterwards, the peptides were separated by reversed phase chromatography with a 155 min non-linear gradient from 1 to 50 % buffer B (0.1 % (v/v) acetic acid in acetonitrile) with a constant flow rate of 0.3 µl/min and injected online into the mass spectrometer. The 20 most abundant precursor ions were selected for collision-induced dissociation (CID) fragmentation after a survey scan in the Orbitrap with a resolution of 60,000 and activated lockmass correction. MS/MS scans were recorded in the dual pressure linear ion trap after fragmentation was performed for 10 msec with a normalized collision energy of 35.

Data analysis was performed according to Bonn et al. [21,17]. In brief, database searching was done with Sorcerer-SEQUEST 4 (Sage-N Research, Milpitas, USA). After data extraction from raw files, the *.dta files were searched with Sequest against a target-decoy database with a set of common laboratory contaminants. A non-redundant database for peptide/protein searches was created from the genome sequences of the seven study isolates and the genome sequence of the *E. cloacae* type strain ATCC 13047 as downloaded from Uniprot (http://www.uniprot.org; 23rd of October 2015). The used database includes protein sequences that differ in at least 1 amino acid, and it contains 30486 proteins in total. Only strict tryptic peptides with up to two missed cleavages were used for the database search. Fixed modifications were not considered. Oxidation of methionine and carbamidomethylation of cysteine were considered as variable modifications. Mass tolerance for precursor ions was set to 10 ppm, and for fragment ions to 0.5 Da. Validation of the MS/MS-based peptide and protein identification was performed with Scaffold v.4.4.1.1 (Proteome Software, Portland, USA). Peptide identifications were only accepted if they exceeded the following specific database search engine thresholds: the SEQUEST identifications required at least deltaCn scores of > 0.1 and XCorr scores of > 2.2, 3.3 and 3.7 for doubly, triply and all higher charged peptides, respectively. Protein identifications (Supplemental Table S2) were accepted if at least 2 identified peptides were detected with above mentioned filter criteria in 2 out of 3 biological replicates. This resulted in a false-positive discovery rate (FDR) below 0.2% on protein level as was verified by a search against a concatenated target-pseudoreversed decoy database. All MS data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD007113 [22].

## Ethics statement

The bacterial isolates used for the present analyses were collected in the course of routine diagnostics and infection prevention control. Oral consent for the use of such clinical samples for research purposes is routinely obtained upon patient admission to the UMCG, in accordance with the guidelines of the Medical Ethics Committee of the University Medical Center Groningen. All experiments were performed in accordance with the guidelines of the Declaration of Helsinki and the institutional regulations, and all samples were anonymized.

## Funding

This work was supported by the Graduate School for Medical Sciences of the University of Groningen (to S.N. and S.G.), the People Programme (Marie Skłodowska-Curie Actions) of the European Union's Horizon 2020 Programme under REA grant agreement (no. 642836 to S.G., D.B and J.M.v.D.), the Deutsche Forschungsgemeinschaft (SFB/TRR 34 framework to D.B., F.B. and A.O.), the German Federal Ministry of Education and Research (Septomics to F.B.), and the Fundamental Research Funds for the Central Universities (2016FZA7008 to KZ). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Disclosure of Potential Conflicts of Interest

J.W.A.R. is being employed by the company IDbyDNA. The other authors declare that they have no financial and non-financial competing interests in relation to the documented research.

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